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Title

Dietary nitrate supplementation enhances high-intensity running performance in moderate normobaric hypoxia, independent of aerobic fitness

Authors

Oliver Michael Shannon^a, Lauren Duckworth^a, Matthew John Barlow^a, David Woods^{ab}, Jose Lara^c, Mario Siervo^d, John Paul O'Hara^a

Affiliations

^a Research Institute for Sport, Physical Activity, and Leisure, Leeds Beckett University, Leeds, LS163QS, United Kingdom

^b Defence Medical Services, Royal Centre for Defence Medicine, Birmingham, B152TH United Kingdom

^c Department of Applied Sciences, Northumbria University, Newcastle upon Tyne, NE18ST, United Kingdom

^d Institute for Aging and Health, University of Newcastle, Newcastle upon Tyne, NE45PL, United Kingdom

Corresponding Author

Oliver Michael Shannon O.Shannon@LeedsBeckett.ac.uk

Research Institute for Sport, Physical Activity, and Leisure

Leeds Beckett University

Leeds

LS6 3QS

United Kingdom

Abstract

Nitrate-rich beetroot juice (BRJ) increases plasma nitrite concentration, lowers the oxygen cost ($\dot{V}O_2$) of steady-state exercise and improves exercise performance in sedentary and moderately-trained, but rarely in well-trained individuals exercising at sea-level. BRJ supplementation may be more effective in a hypoxic environment, where the reduction of nitrite into nitric oxide (NO) is potentiated, such that well-trained and less well-trained individuals may derive a similar ergogenic effect. We conducted a randomised, counterbalanced, double-blind placebo controlled trial to determine the effects of BRJ on treadmill running performance in moderate normobaric hypoxia (equivalent to 2500 m altitude) in participants with a range of aerobic fitness levels. Twelve healthy males ($\dot{V}O_{2\max}$ ranging from 47.1 - 76.8 ml·kg⁻¹·min⁻¹) ingested 138 ml concentrated BRJ (~ 15.2 mmol nitrate) or a nitrate-deplete placebo (PLA) (~ 0.2 mmol nitrate). Three hours later, participants completed steady-state moderate intensity running, and a 1500 m time-trial (TT) in a normobaric hypoxic chamber ($F_{I}O_2$ ~15 %). Plasma nitrite concentration was significantly greater following BRJ versus PLA 1 hour post supplementation, and remained higher in BRJ throughout the testing session ($p < 0.01$). Average $\dot{V}O_2$ was significantly lower (BRJ: 18.4 ± 2.0 , PLA: 20.4 ± 12.6 ml·kg⁻¹·min⁻¹; $p = 0.002$), whilst arterial oxygen saturation (S_aO_2) was significantly greater (BRJ: 88.4 ± 2.7 , PLA: 86.5 ± 3.3 %; $p < 0.001$) following BRJ. BRJ improved TT performance in all 12 participants by an average of 3.2 % (BRJ: 331.1 ± 45.3 vs. PL: 341.9 ± 46.1 s; $p < 0.001$). There was no apparent relationship between aerobic fitness and the improvement in performance following BRJ ($r^2 = 0.05$, $p > 0.05$). These findings suggests that a high nitrate dose in the form of a BRJ supplement may improve running performance in individuals with a range of aerobic fitness levels conducting moderate and high-intensity exercise in a normobaric hypoxic environment.

Key words: Nitric Oxide, Nitrate, Exercise performance, Hypoxia

1. Introduction

Supplementation with dietary nitrate via nitrate-rich foods or nitrate salts elicits an array of potentially beneficial physiological changes. These include, but are not restricted to: lower blood pressure [1–3], reduced O_2 consumption ($\dot{V}\text{O}_2$) during steady-state exercise [4–6], attenuated muscle metabolic perturbations [7,8], and enhanced muscle force production [9,10]. These effects are not directly attributable to the nitrate anion, which is relatively inert [11]. Instead, the benefits of nitrate supplementation appear to be related to an increased production of the multifunctional signalling molecule nitric oxide (NO). Ingested nitrate is reduced to nitrite by symbiotic bacteria residing predominantly on the dorsal surface of the tongue [12,13]. A portion of the nitrite is converted into NO in the acidic environment of the stomach [14,15], but the majority enters systemic circulation where it may be reduced to NO and other bioactive nitrogen oxides in the blood and tissue via various nitrite reductases [11].

Increasing NO bioavailability via nitrate supplementation has been reported to improve exercise time to exhaustion (TTE) or time-trial (TT) performance in the majority [5,16–18], but not all [23,24] previous investigations in untrained and moderately-trained individuals. Conversely, well-trained participants ($\dot{V}\text{O}_{2\text{max}} > 60 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) are typically less responsive to dietary nitrate supplementation [17,19–21,25–27], even when very high nitrate doses ($\sim 19.5 \text{ mmol}$) are administered [28]. Highly-trained individuals are known to possess elevated baseline nitrate/ nitrite reserves [29], may habitually consume large amounts of nitrate, subsequent to their increased calorie consumption [30], and exhibit greater presence and activity of the NOS enzymes, relative to the untrained [31]. Such differences may reasonably be expected to lessen the ergogenic effect of nitrate supplementation [30].

The reduction of nitrite to NO, and hence the capacity to influence NO mediated physiological processes, is potentiated when muscle pH [32] and PO_2 [33] decline. Conversely, the L-arginine NOS pathway becomes dysfunctional with low O_2 tensions and pH [33]. Such cellular conditions are likely to be especially prevalent during exercise in a hypoxic environment [8,34], such as experienced on ascent to terrestrial altitude. This suggests that nitrate supplementation may be more likely to enhance NO generation and therefore improve performance in hypoxia relative to normoxia. Moreover, an improvement in mitochondrial [35] and/ or muscle contractile efficiency [7] subsequent to nitrate supplementation which reduces $\dot{V}\text{O}_2$ is likely to be especially advantageous when O_2 availability is low. This is supported by the findings of a recent study, in which nitrate supplementation improved tolerance to severe intensity cycling in hypoxia ($F_{\text{I}}\text{O}_2$ 13.1 %) but not normoxia [36]. However, in other studies, nitrate supplementation has been shown both to improve [8,22,37] and have no effect [38–40] on hypoxic exercise performance. These equivocal findings may be attributed to differences in the training status of participants, exercise protocol, supplementation protocol, and hypoxic stimuli applied, and warrant further exploration.

Evidence from murine models suggests that the effects of dietary nitrate supplementation may be fibre type specific, increasing muscle perfusion and oxygenation [41,42], and enhancing calcium handling and force production [9] in type II muscle fibres only, as may be recruited during high-intensity exercise (for review, see Jones et al. [43]). Moreover, nitrate supplementation has been demonstrated to elicit beneficial effects when the contribution of type II muscle towards force generation is high, including at fast but not slow muscle

contraction frequencies [44], and during the transition from moderate to severe but not unloaded to moderate intensity exercise [45].

It is possible that nitrate supplementation is especially effective when conducting high-intensity exercise in a hypoxic environment, such that individuals across a range of aerobic fitness levels may derive a similar performance enhancing benefit from dietary nitrate supplementation. This has considerable relevance, given the widespread popularity of travel to high-altitude (i.e. hypobaric hypoxia) each year for recreational (e.g. hiking, skiing, mountaineering) and sporting (e.g. training camps, high-altitude running events, and cycle mountain stages) purposes in individuals with a range of different fitness levels. Therefore, the purpose of this study was to assess the effect of nitrate supplementation on physiological functioning and TT performance in moderate normobaric hypoxia (equivalent to 2500 m) in individuals with varying aerobic fitness levels ($\dot{V}O_{2\max}$). We hypothesised that: (1) dietary nitrate supplementation would enhance exercise performance in hypoxia and (2) this effect would be consistent across a range of aerobic fitness levels.

2. Methods

2.1 Participants

Twelve healthy men aged 24.4 ± 4.3 years, with a body mass of 76.4 ± 9.6 kg, stature of 181.6 ± 6.4 cm, and maximal O_2 uptake ($\dot{V}O_{2\max}$) at sea-level (normoxia) of 62.1 ± 9.3 ml·kg⁻¹·min⁻¹ (range: 47.1 to 76.8 ml·kg⁻¹·min⁻¹) and simulated altitude (2500 m) of 52.2 ± 7.5 ml·kg⁻¹·min⁻¹ (range: 40.8 to 61.8 ml·kg⁻¹·min⁻¹) volunteered to take part in this study. Participants were recruited across a range of aerobic fitness levels to assess the relationship between $\dot{V}O_{2\max}$ and the change in hypoxic exercise performance consequent to nitrate supplementation. The participant cohort comprised six competitive runners / triathletes (all with experience racing over 1500 m), four individuals who regularly took part in recreational sport but were not competitive athletes, and two individuals who were physically active, yet did not take part in any sport. The study received institutional ethical approval and was conducted in accordance with the Declaration of Helsinki. Participants provided written informed consent prior to testing.

2.2 Overview

Participants visited the laboratory on five occasions within a six week period. On the first visit to the laboratory, participants completed an incremental running test to volitional exhaustion in normoxia to determine $\dot{V}O_{2\max}$. The values obtained were used to define training status. Each subsequent session involved exercise in a normobaric hypoxic chamber (TISS, Alton, UK, and Sporting Edge, Sherfield on Loddon, UK) situated at ~ 113 m above sea-level at a simulated altitude of 2500 m ($F_{iO_2} \sim 15.0$ %). F_{iO_2} was adjusted daily based around fluctuations in barometric pressure, and accounting for 47 mmHg water vapour pressure [46]. Ambient air temperature and relative humidity were maintained at 20 °C and 50 % respectively. On the second visit to the laboratory, participants completed an incremental running test in hypoxia, to define altitude specific $\dot{V}O_{2\max}$, and elucidate suitable relative exercise intensities for the experimental trials. The third visit involved a familiarisation session, replicating the experimental trials but without any intervention. The fourth and fifth visits constituted the experimental trials. Experimental trials were preceded by the consumption of 138 ml concentrated nitrate-rich (BRJ) (~ 15.2 mmol nitrate) or nitrate-deplete (PLA) (~ 0.2 mmol

nitrate) beetroot juice (BEET IT, James White Drinks Ltd., Ipswich, UK), administered in a randomised double-blind cross-over design three hours before exercise. Participants were asked to abstain from intense exercise, alcohol, and caffeine for 24 hours prior to each trial. Antibacterial mouthwash and chewing gum, known to ablate the oral bacteria responsible for nitrate reduction into nitrite, was also avoided [47].

2.3. Preliminary trials

A two-part incremental running test was conducted on a motorised treadmill (Woodway, Cranlea, Birmingham, UK) [48], once in normoxia and once in normobaric hypoxia. Participants completed five to eight sub-maximal stages lasting three minutes, separated with one minute recovery periods. Starting speed was determined based around perceived participant fitness. Running speed was increased by 1 km·h⁻¹ each stage, and a 1 % treadmill gradient was applied to approximate the energetic cost of outdoor running [49]. Finger-tip blood samples were obtained between stages to determine blood lactate concentration (YSI 2300 STAT plus, Yellow Springs, Ohio). Exercise was ceased when blood lactate concentration reached 4 mM. The second phase of the test commenced following 5 minutes recovery. A fixed running speed equal to the final speed obtained during the first part of the test, minus 2 km·h⁻¹ was applied. The gradient was increased by 1 % every minute, until volitional exhaustion. Respiratory variables were monitored continuously via an online gas analysis system, calibrated before each trial according to the manufacturer's instructions (MedGraphics Ultima CPX, MGC Diagnostics, MN, USA).

2.4. Experimental trials

2.4.1. Protocol

On the morning of each experimental session, participants arrived at the laboratory between 7 and 9 am following an overnight fast. A cannula was fitted by a trained phlebotomist into a vein in the arm to enable repeated blood sampling. Participants then ingested BRJ or PLA within a 5 minute period and consumed a standardised breakfast (360 kcal, carbohydrates 62 %, fat 22 %, protein 16 %) within a 10 minute period. They then rested in normoxia for 2.5 hours, during which time water was permitted *ad libitum*. Participants then entered the hypoxic chamber, where they rested for a further 30 minutes. Exercise commenced 3 hours post-supplementation, and included 2 x 15 minute bouts of steady-state running at 45 % and 65 % altitude-specific $\dot{V}O_{2max}$, each followed by a 5 minute recover period. A 1500 m TT then commenced. Participants ran at a speed approximating 80 % altitude-specific $\dot{V}O_{2max}$ for 30 seconds as a 'rolling start', before the TT commenced. Participants were asked to run the 1500 m TT as fast as possible. Running speed and time were not visible during the TT, although participants were informed of the distance they had covered at 200 m intervals. The treadmill gradient was set to 1 % throughout exercise [49]. We have previously demonstrated excellent reliability of this performance test (CV < 1 %)[50]. Participants rested in normobaric hypoxia for 10 minutes following TT, after which they were free to leave.

2.4.2. Measurements

Ten blood samples were drawn throughout each experimental trial, comprising two, 4 ml lithium heparin containing vacutainers (Becton Dickinson, Plymouth, UK) for later determination of plasma nitrate and nitrite. Measurement points included pre-supplementation, 30, 60, 90, 120, 150 (pre-hypoxic exposure), and 180 (pre-exercise) minutes post-

supplementation, following each bout of steady-state exercise, and immediately post-TT. Blood pressure (BP) of the brachial artery was measured using an automated sphygmomanometer (Omron Healthcare Ltd., Kyoto, Japan) pre-supplementation, pre-hypoxic exposure, pre-exercise, and 5 minutes post-TT. Mean arterial pressure (MAP) was calculated as $1/3 \cdot \text{systolic pressure} + 2/3 \cdot \text{diastolic pressure}$ [51]. At the same time points, a measure of exhaled NO was also recorded, using a hand-held electrochemical analyser (NObreath, Bedfont Scientific Ltd., UK). Four measures were obtained for BP and exhaled NO, and the mean value of the final three measurements was used for data analysis. Heart rate (HR) was measured via a chest worn heart-rate monitor strap (Polar Electro, Oy, Finland) pre-supplementation, pre-hypoxic exposure, pre-exercise, during the final 2 minutes of each steady-state exercise bout, and immediately post-TT. Arterial O₂ saturation (S_aO₂) was monitored via pulse oximetry (Nellcor, Medtronic, Minneapolis, MN) pre-hypoxic exposure, pre-exercise, during the final 2 minutes of each steady-state exercise bout, and immediately post-TT. Expired gas was monitored as previously described for 10 minutes pre-exercise, and during steady-state exercise. Data obtained during the final 5 minutes of rest and each steady-state exercise bout was averaged and used for subsequent analysis. Perceptions of exertion were also monitored during the final 2 minutes of each steady-state exercise bout and immediately post-TT using a 15 point (6 – 20) ratings of perceived exertion (RPE) scale [52].

2.5. Assessment of NO blood metabolites

2.5.1. Blood handling

Blood samples were centrifuged at 5000 rpm for 3 minutes immediately post-collection. Plasma (1 ml) was drawn from each vacutainer into opaque cryotubes (Argos Technologies, IL, USA), each containing 6.5 mM N-ethylmaleimide (NEM) and 0.1 mM Diethylenetriaminepentaacetic acid (DTPA). NEM and DTPA were added to prevent the interchange between NO metabolites [53]. Cryotubes were immediately placed in a freezer at – 80 °C, for later analysis of nitrate and nitrite. 1 ml was also extracted from each 70 ml beetroot juice ‘shot’ prior to administration, and frozen in untreated opaque cryotubes at – 80 °C for subsequent determination of nitrate concentration.

2.5.2. Ozone-based chemiluminescence

Plasma nitrate and nitrite concentration, and the nitrate content of administered beetroot juice were measured by ozone-based chemiluminescence as per the manufacturer’s instructions (Sievers NOA 280i, Analytix, UK). Briefly, nitrite was determined by addition of samples to 0.17M sodium iodide in glacial acetic acid under nitrogen at room temperature. Sodium iodide in acetic acid has the capacity to convert nitrite to NO, but is unable to reduce any higher oxides of nitrogen such as nitrate and thus is relatively specific for nitrite. To obtain concentration of total plasma nitrogen oxides (NO_x), we used the same apparatus with a stronger reducing agent vanadium chloride (0.1M) in hydrochloric acid (1M) at 95 °C. These stronger conditions reduce the sum of all nitrogen oxides, which is predominantly nitrate (μM) but also includes both nitrite (nM) and nitrosothiols (nM). Nitrate concentration was calculated by subtraction of the nitrite from NO_x.

2.6. Statistical analysis

Data analysis was conducted using SPSS version 22. An α level of $p < 0.05$ was accepted for significance. Normality was assessed using the Shapiro-Wilk test. Non-normal data was log-

transformed (\log_{10}) prior to analysis. Physiological data was compared between trials using a two-way (time and condition) ANOVA. To adjust for asphericity, the Greinhouse Geisser correction was applied for $\varepsilon < 0.75$, and the Huynh-Feldt correction was adopted for $\varepsilon > 0.75$. Post-hoc analysis was conducted using t-tests with a Bonferroni adjustment. A paired t-test was used to compare TT performance between conditions. The square of Pearson's correlation coefficient (r^2) was used to explicate the relationship between variables. A statistical spreadsheet was also used to derive a qualitative probabilistic inference for performance data [54]. One of the following verbal descriptors was assigned to describe the likelihood of a practically beneficial effect of BRJ on performance: $< 0.5\%$, 'almost certainly not'; $0.5 - 5\%$, 'very unlikely not'; $5 - 25\%$, 'unlikely'; $25 - 75\%$, 'possibly'; $75 - 95\%$, 'likely'; $95 - 99.5\%$, 'very likely'; $> 99.5\%$, 'almost certainly'. Data are presented as means \pm SD, unless otherwise stated.

3. Results

3.1. Plasma nitrate and nitrite and exhaled NO

Plasma nitrate, nitrite and exhaled NO data are presented in Figure 1. Pre-supplementation plasma nitrate concentration was no different between BRJ ($28.8 \pm 14.3 \mu\text{mol}\cdot\text{L}^{-1}$) and PLA ($30.1 \pm 29.4 \mu\text{mol}\cdot\text{L}^{-1}$) ($p > 0.05$). There was a marked increase in plasma nitrate concentration (i.e. a condition effect) in BRJ ($493.0 \pm 174.4 \mu\text{mol}\cdot\text{L}^{-1}$) versus PLA ($32.5 \pm 34.6 \mu\text{mol}\cdot\text{L}^{-1}$, $p < 0.01$). Significant effects for time ($p < 0.001$) and time * condition interaction effects ($p < 0.001$) were observed. Post-hoc analysis revealed significantly elevated plasma nitrate concentration in BRJ compared to PLA and pre-supplementation baseline values, 30 minutes post-supplementation, and at all subsequent measurement points ($p < 0.001$). Peak plasma nitrate concentration occurred on average approximately 2 hours post-supplementation (BRJ = 594.1 ± 90.5 vs. PLA = $33.9 \pm 35.6 \mu\text{mol}\cdot\text{L}^{-1}$, $p < 0.001$). Plasma nitrate concentration was unchanged relative to pre-supplementation in PLA ($p > 0.05$).

Pre-supplementation plasma nitrite concentration did not differ between BRJ ($87.5 \pm 65.7 \text{ nmol}\cdot\text{L}^{-1}$) and PLA ($91.7 \pm 70.8 \text{ nmol}\cdot\text{L}^{-1}$) ($p > 0.05$). Conversion of nitrate into nitrite was evident, with a significant effect of condition on plasma nitrite concentration (BRJ: 468.0 ± 252.5 vs. $105.4 \pm 57.5 \text{ nmol}\cdot\text{L}^{-1}$, $p < 0.001$). Significant effects for time ($p < 0.001$) and time * condition interaction effects ($p < 0.001$) were observed. Post-hoc analysis revealed that plasma nitrite was significantly greater in BRJ versus PLA and pre-supplementation baseline values, 1 hour post-supplementation ($p < 0.01$), and remained higher in BRJ across all subsequent measurement points ($p < 0.001$). Peak plasma nitrite concentration occurred on average approximately 2.5 hours post-supplementation (BRJ = 660.4 ± 265.0 vs. PLA = $109.7 \pm 61.0 \text{ nmol}\cdot\text{L}^{-1}$, $p < 0.001$). Plasma nitrite concentration was unchanged relative to pre-supplementation values in PLA ($p > 0.05$).

Pre-supplementation concentration of exhaled NO was no different between BRJ ($32.1 \pm 34.7 \text{ p.p.b.}$) and PLA ($36.8 \pm 31.5 \text{ p.p.b.}$) ($p > 0.05$). There was a significant condition effect on exhaled NO (BRJ = 54.1 ± 39.8 vs. PLA = $33.6 \pm 31.1 \text{ p.p.b.}$, $p = 0.002$). Significant effects for time ($p = 0.021$) and time * condition interaction effects ($p = 0.002$) were observed. Post-hoc analysis revealed exhaled NO was significantly elevated in BRJ compared with PLA and pre-supplementation values at all post-supplementation measurement points ($p < 0.05$), but were unchanged in PLA ($p > 0.05$).

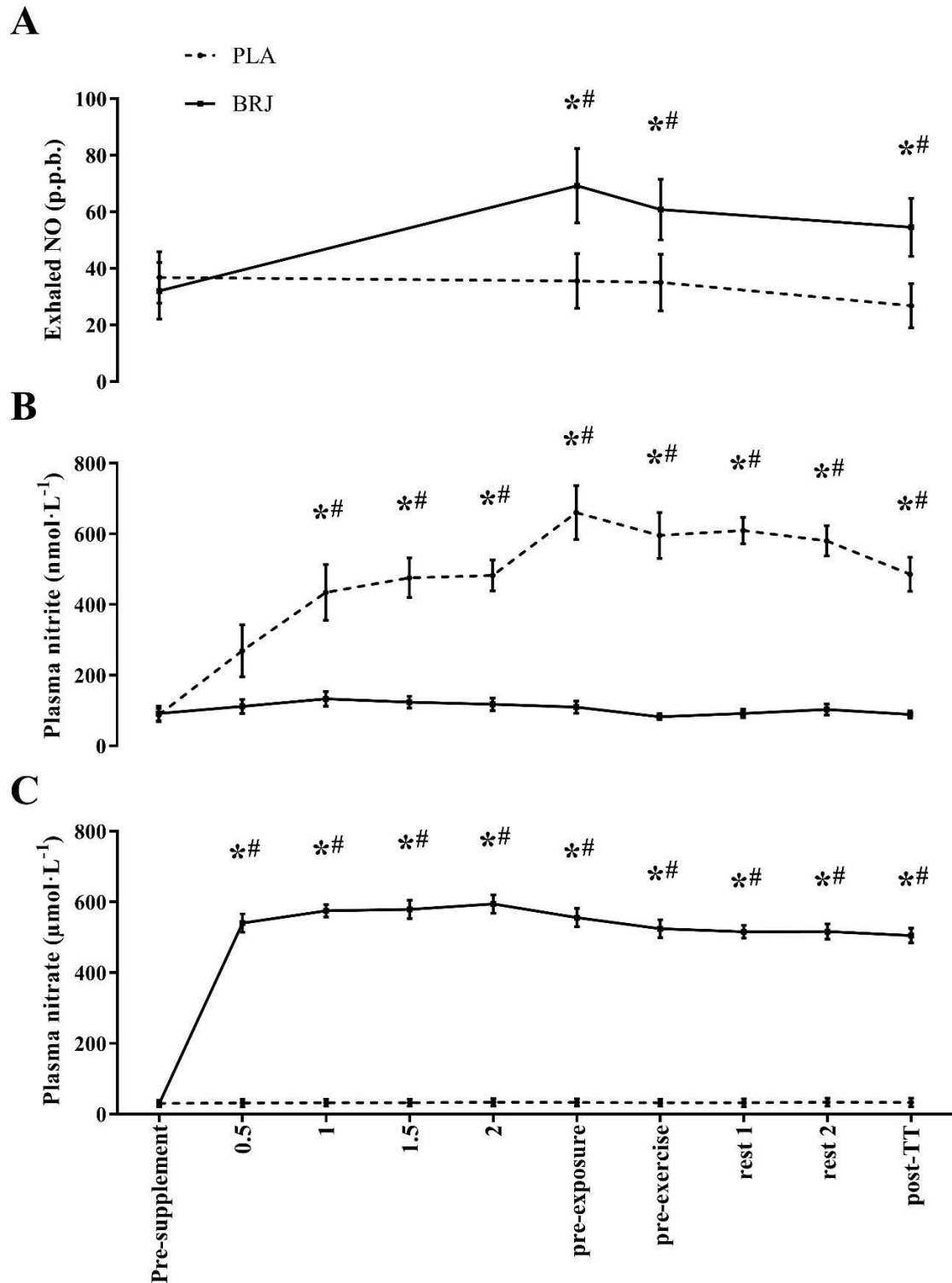


Figure 1 Exhaled NO (A), Plasma nitrite (B) and plasma nitrate (C) throughout experimental trials in the placebo (PLA) (dashed lines) and nitrate-rich beetroot juice (BRJ) (solid lines) conditions. Values are mean \pm SEM. * Time points significantly different between BRJ and PLA conditions ($p < 0.05$). # Time points significantly different from baseline in the BRJ condition.

3.2. Perceived exertion and cardio-respiratory variables

Data for RPE, HR, MAP, $\dot{V}O_2$, and S_aO_2 is presented in Table 1. There was a significant condition effect for RPE (BRJ: 13 ± 4 vs. PLA: 14 ± 4 , $p = 0.037$), and a clear effect of time

on RPE ($p < 0.001$), although no time * condition interaction effects were observed ($p = 0.152$). There was a significant time effect on HR ($p < 0.001$), but no condition ($p = 0.495$) or time * condition interaction effects ($p = 0.383$) were observed. MAP showed a significant effect of condition (BRJ: 83 ± 7 vs. PLA: 86 ± 7 mmHg, $p = 0.006$), but no time ($p = 0.187$) or time * condition interaction effects ($p = 0.646$). There was a significant effect of condition on $\dot{V}O_2$ (BRJ: 18.4 ± 12.0 vs. 20.4 ± 12.6 ml·kg⁻¹·min⁻¹, $p = 0.002$). Likewise, there were significant effects of time ($p < 0.001$) and time * condition interaction effects ($p = 0.005$) on $\dot{V}O_2$. Post-hoc analysis revealed significantly lower $\dot{V}O_2$ in BRJ during exercise at 45 % ($p = 0.014$) and 65 % ($p = 0.002$) $\dot{V}O_{2max}$. There was a significant effect of condition (BRJ: 88 ± 3 vs. PLA: 87 ± 3 %, $p < 0.001$), time ($p < 0.001$) and time * condition interaction effects ($p = 0.034$) on S_aO_2 . Post-hoc analysis revealed significant differences in S_aO_2 during the pre-exercise rest period ($p = 0.012$) and during exercise at 45 % $\dot{V}O_{2max}$ ($p = 0.003$). There was no significant relationship between the reduction in $\dot{V}O_2$ and increase in S_aO_2 consequent to BRJ ($r^2 = 0.13$, $p = 0.242$).

Table 1 Ratings of perceived exertion, heart rate, mean arterial blood pressure, oxygen consumption, and arterial oxygen saturation throughout experimental trials in the placebo (PLA) and nitrate-rich beetroot juice (BRJ) conditions

Variable and condition	Arrival	Pre-exposure	Pre-exercise	45 % $\dot{V}O_{2max}$	65 % $\dot{V}O_{2max}$	TT
RPE						
PLA				10 ± 1	12 ± 1	19 ± 1
BRJ ^a				9 ± 1	12 ± 1	19 ± 1
HR (b·min ⁻¹)						
PLA	61 ± 8	60 ± 9	58 ± 9	107 ± 16	146 ± 13	183 ± 9
BRJ	58 ± 9	60 ± 8	59 ± 7	105 ± 18	144 ± 12	183 ± 8
MAP (mmHg)						
PLA	87 ± 8	86 ± 5	85 ± 7			85 ± 10
BRJ ^a	87 ± 10	81 ± 10	83 ± 7			82 ± 5
$\dot{V}O_2$ (ml·kg ⁻¹ ·min ⁻¹)						
PLA			4.8 ± 1.0	21.4 ± 6.0	33.7 ± 4.3	
BRJ ^a			4.5 ± 0.7	19.2 ± 5.9^b	31.4 ± 3.9^b	
S_aO_2 (%)						
PLA		98 ± 1	91 ± 3	85 ± 2	81 ± 3	78 ± 7
BRJ ^a		97 ± 2	94 ± 2^b	88 ± 2^b	83 ± 2	80 ± 6

^a denotes significant difference overall (condition effect) versus PLA

^b denotes significant difference (time * condition interaction effect) versus PLA

3.5. TT performance

BRJ improved 1500 m TT performance by 3.2 % (BRJ = 331.1 ± 45.3 s) versus PLA (341.9 ± 46.1 s, $p < 0.001$). Magnitude based inferences [54] indicated that the true value of the effect would ‘almost certainly’ be practically beneficial to an athlete. All 12 participants completed the TT quicker following BRJ ingestion (Figure 2.), with the range of improvement between

1.1 and 20.2 s. There was no significant correlation between $\dot{V}O_{2\max}$ measured at sea-level ($r^2 = 0.05$; $p = 0.48$) nor simulated-altitude ($r^2 = 0.05$, $p = 0.46$) and the change in performance following BRJ supplementation. The observed r^2 value indicates that 5 % of the 1500 m TT variability can be explained in terms of differences in $\dot{V}O_{2\max}$. There was no significant correlation between the change in $\dot{V}O_2$ ($r^2 = 0.04$, $p = 0.84$) nor S_aO_2 ($r^2 = 0.001$, $p = 0.90$) and the change in performance following BRJ.

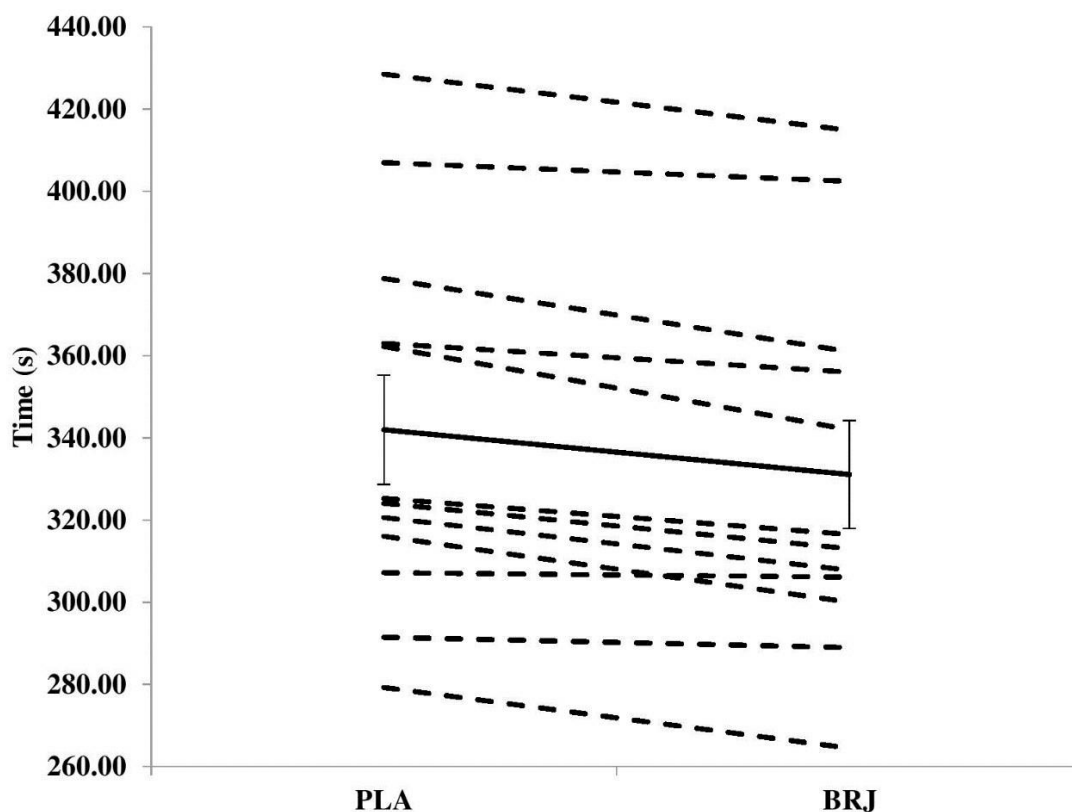


Figure 2 1500 m TT performance following ingestion of placebo (PLA) or nitrate-rich beetroot juice (BRJ).

4. Discussion

Nitrate-rich beetroot juice has emerged as a popular ergogenic aid, although previous research suggests that this supplement has limited effects in well-trained individuals ($\dot{V}O_{2\max} > 60 \text{ ml} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$). In contrast, the results of this study suggest that nitrate-rich beetroot juice may reduce the O_2 cost of exercise, elevate S_aO_2 , and enhance 1500 m TT performance in individuals across a range of different fitness levels exercising in moderate-normobaric hypoxia. These data have relevance for the thousands of recreational and competitive athletes ascending to altitude each year for sporting purposes.

4.1. Plasma nitrate, nitrite, and exhaled NO

Consistent with previous investigations, plasma nitrate and nitrite, and exhaled NO concentrations were significantly elevated following dietary nitrate supplementation [1,2,5,8,55,22,39,38], signifying an increase in the ‘substrates’ available for NO generation via the nitrate-nitrite-NO pathway [11]. Peak plasma nitrite concentration was 602 % higher in

BRJ versus PLA, similar to Arnold et al. [38] (775 %) (~ 7 mmol nitrate), but considerably greater than reported by Muggeridge et al. [22] (134 %) (~ 5 mmol nitrate) and Vanhatalo et al. [8] (150 %) (~ 9.3 mmol). Importantly, all participants increased plasma nitrite concentration by substantially greater than 30 % - a cut off proposed by Wilkerson [20] for identifying 'non-responders'. This may, in part, be a consequence of the high nitrate dose administered, and may help explain the consistent ergogenic effect observed in this study. Nevertheless, there was substantial inter-individual variability in baseline plasma nitrite concentration (range: 40 – 300 nmol·L⁻¹), the magnitude of the increase in plasma nitrite concentration post-supplementation (Δ range: 160 – 1335 nmol·L⁻¹), and the time at which peak plasma nitrite concentration occurred (1 – 3 hours post-supplementation). A variable response to nitrate supplementation has been well-reported in the literature [56]. Inter-individual differences could not be explained by participant aerobic fitness in this study. Genetic factors, chronobiology, variations in the quantity and/or taxa of oral nitrate reducing bacteria, and study protocol variables may be important in explaining this phenomenon, and require further investigation [56].

4.2. Effect of nitrate supplementation on cardiorespiratory variables and RPE

In the present study, $\dot{V}O_2$ did not differ significantly between BRJ and PLA during rest in hypoxia, but was significantly lower in BRJ versus PLA during steady-state exercise. These findings are in line with most previous investigations [4,5,22,37], and similar to the results of a recent meta-analysis [6]. The physiological mechanisms underlying the decreased $\dot{V}O_2$ subsequent to nitrate supplementation have not been fully elucidated, but may be accounted for by: a) an improvement in the efficiency of mitochondrial respiration [35] and/or b) enhanced efficiency of muscle force production [7].

Larsen and co-workers [35] reported an improvement in the phosphate/O₂ (P/O) ratio (i.e. the amount of ATP produced per mole O₂ consumed) in mitochondria harvested from the vastus lateralis of healthy men following three days supplementation with sodium nitrate (0.1 mmol·kg⁻¹·d⁻¹). This effect was attributed to decreased proton leak during oxidative phosphorylation, and was associated with a reduced expression of the ATP/ADP translocase protein (ANT) and a tendency towards downregulation of uncoupling protein 3 (UCP3). The change in the mitochondrial P/O ratio correlated with the reduced *in vivo* $\dot{V}O_2$ measured during moderate-intensity cycle ergometry. In contrast to the findings of Larsen et al. [47] with sodium nitrate, a recent investigation by Whitfield and colleagues [57] reported no change in mitochondrial efficiency despite a reduced $\dot{V}O_2$, following seven days supplementation with nitrate-rich beetroot juice (26 mmol·d⁻¹). There is presently no explanation as to why sodium nitrate and nitrate-rich beetroot juice may have different effects on the mitochondria, and direct comparison is therefore warranted to rule out other methodological differences [58]. Nevertheless, alterations in mitochondrial efficiency are not necessarily required to explain the reduction in $\dot{V}O_2$ reported in this study following BRJ supplementation.

Bailey et al. [7] reported reduced intramuscular PCr, ADP, and P_i perturbations and lower $\dot{V}O_2$ during leg extension exercise following nitrate-rich beetroot juice supplementation (5.1 mmol·d⁻¹ x 6 days), reflecting a lower ATP turnover for a given work rate. It was suggested that the decreased ATP turnover occurring after nitrate supplementation might be related to a reduced ATP requirement for actin-myosin cross-bridge cycling and/or calcium handling,

given previous reports that NO slows actin-myosin cross-bridge cycling [59,60] and inhibits calcium-ATPase activity [61,62]. Interestingly, a recent murine model investigation reported greater force production, and increased expression of the calcium handling protein calsoquestrin 1 and the dihydropyridine receptor in type II muscle fibres following nitrate supplementation [9]. It was speculated that such effects in humans may allow muscle activation at a lower frequency for an equivalent force production, lowering motor unit recruitment and concomitantly the ATP cost of exercise.

In the present study, S_aO_2 was significantly elevated overall in BRJ compared to PLA. These findings are similar to those of Masschelein and colleagues [37] who reported a significant increase in S_aO_2 during cycle ergometry exercise in severe hypoxia ($F_I O_2$ 11 %) following 6 days nitrate supplementation ($\sim 5 \text{ mmol} \cdot \text{d}^{-1}$). Tissue oxygenation in the vastus lateralis was also elevated following nitrate supplementation in that study. These findings are supportive of the notion that nitrate supplementation may reduce muscle O_2 consumption during exercise in hypoxia, presumably via the aforementioned mitochondrial [35] and/or muscle [7] alterations that also manifest as a reduced $\dot{V}O_2$. Interestingly, Arnold et al. [38] found that runners identified as ‘responders’ to nitrate supplementation during a 10,000 m TT in moderate hypoxia ($F_I O_2$ 15.4 %) typically experienced a lower S_aO_2 during the placebo TT than ‘non-responders’ (82 vs 84 %). This suggests that the S_aO_2 response to hypoxia may moderate the performance effects of nitrate supplementation, possibly due to greater nitrite reduction into NO at lower O_2 tensions [33]. In this study, there was no apparent relationship between average nor post-TT S_aO_2 values and the percentage improvement in performance ($r^2 = 0.05$). However, S_aO_2 reached lower values in the present investigation (post TT PLA: $77.8 \pm 6.9 \%$) than observed by Arnold et al. [38], presumably due to the shorter higher-intensity TT, which may be important.

Interestingly, RPE was significantly lower overall in BRJ versus PLA, suggesting reduced overall physiological ‘strain’ [52], which may in part be reflective of the lower $\dot{V}O_2$ and/or elevated S_aO_2 with BRJ. In normoxia, Murphy and colleagues [63] reported lower RPE during the first mile of a 5 km running TT, although others have reported no effect of nitrate supplementation on RPE during sub-maximal and maximal exercise in hypoxia following nitrate supplementation [37,38].

4.3. Effect of nitrate supplementation on running performance in hypoxia

The main finding of the present study was that BRJ improved 1500 m running performance in moderate normobaric hypoxia by 3.2 % versus PLA. Magnitude based inferences suggested an ‘almost certain’ chance that the true value of the effect would be practically beneficial. In agreement with our hypothesis, there was no apparent relationship between the change in exercise performance post-BRJ supplementation and $\dot{V}O_{2\text{max}}$ measured in normoxia nor hypoxia, suggesting a similar effect of BRJ on TT performance across a range of fitness levels under these experimental conditions.

A number of previous investigations have confirmed the beneficial effect of nitrate supplementation on TTE [8,24,37] and TT performance [22] in hypoxia. Conversely, studies in well-trained individuals exercising in hypoxia have typically found no effect of nitrate supplementation [38–40]. Well-trained individuals exhibit increased presence and activity of

the NOS enzymes [31]; possess higher baseline nitrate/nitrite concentration [29]; may habitually consume high levels of nitrate as a consequence of their large daily energy intake [30]; and experience lower tissue acidosis and hypoxia [20] relative to the untrained. Moreover, a recent investigation in normoxia observed an inverse correlation between aerobic fitness and the response to nitrate supplementation [17]. It is therefore reasonable to assume that adaptations elicited by endurance training may blunt the response to nitrate supplementation. Nevertheless, our results suggest a high training status does not necessarily preclude an ergogenic effect of nitrate supplementation. Instead, it is suggested that individuals across a spectrum of aerobic fitness levels may derive similar performance enhancing benefits of nitrate supplementation under specific experimental conditions, including a hypoxic exercise environment, short-duration high-intensity TT protocol, and high nitrate dose.

Considering generation of NO via the L-arginine NOS pathway is suppressed in hypoxia, yet the nitrate-nitrite-NO pathway is potentiated as O₂ tensions fall [11,33], it is likely that nitrate supplementation is more effective in hypoxia than normoxia. Supportive evidence is provided by Kelly et al. [36], who subjected healthy male participants to identical exercise regimes preceded by the same nitrate supplement strategy (8.4 mmol·d⁻¹ x 3 days), but with exercise varied between normoxia and hypoxia (F_IO₂ 13.1 %). Nitrate supplementation had no effect on severe-intensity cycling TTE in normoxia, but improved TTE by ~ 8.6 % in hypoxia relative to placebo. Further, there is evidence from murine model investigations that nitrate supplementation may enhance tissue blood flow [41,42] and muscle contractile function [9] preferentially in type II muscle fibres. It is therefore possible that nitrate supplementation is more effective during shorter more high-intensity exercise, during which these muscle fibres are more heavily recruited [9,43–45,64], or else in individuals with greater distribution of type II muscle fibres [43]. The time-trial duration employed in this study (< 6 minutes) was considerably less than other investigations (~ 17 - 48 minutes) which have not observed an ergogenic effect of nitrate supplementation in hypoxia [38–40], and may be important. Thus, the combination of a hypoxic exercise environment and high-intensity TT may have maximised the effects of nitrate supplementation.

Finally, there is some evidence of a dose-response to nitrate supplementation [55,65,66], and it is possible that well-trained athletes may require a high nitrate dose to appreciably alter plasma nitrite concentration relative to untrained individuals [30]. Therefore, the high nitrate dose administered in this study relative to investigations which have not observed an effect of nitrate supplementation (~ 5 – 7 mmol) [38–40] may have also contributed towards the consistent ergogenic effect observed here.

4.4. Strengths and limitations

The current study has several strengths. In particular, the broad spectrum of participant aerobic fitness levels allowed us to assess the relationship between $\dot{V}O_{2\max}$ and the improvement in hypoxic exercise performance following nitrate supplementation. Nevertheless, none of our participants were elite athletes nor entirely sedentary, and it is possible that individuals outside of the present fitness range may respond differently to nitrate supplementation. Moreover, we only included healthy male participants, and further research is warranted in other populations who may respond differently to nitrate supplementation [67]. A further strength of this investigation was that the consumption of nitrate-rich foods was not restricted during the study

period. This approach, first employed by Vanhatalo and colleagues [51] and later adopted in several subsequent investigations [25,68], preserves ecological validity and demonstrates that nitrate supplementation can alter physiological functioning and exercise performance when participants are consuming their normal diet.

4.5. Conclusion

The present study reported an increase in plasma nitrite concentration, reduction in steady-state $\dot{V}O_2$, elevation in S_aO_2 , and enhancement of 1500 m running performance in normobaric hypoxia following supplementation with dietary nitrate. Further, this effect did not appear to be related to the aerobic fitness of participants. This suggests that individuals across a range of different aerobic fitness levels may derive a similar performance enhancing benefits of dietary nitrate when consuming a high nitrate dose, and conducting moderate and high-intensity exercise in a hypoxic environment.

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